

AD \_\_\_\_\_

Award Number: DAMD17-02-1-0449

TITLE: The Role of MEKK3 Signaling of Pathway in the Resistance  
of Breast Cancer Cells to TNF-(alpha)-Mediated Apoptosis

PRINCIPAL INVESTIGATOR: Ling Yu, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson  
Cancer Center  
Houston TX 77030

REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050121 028

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> May 2004	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 May 2003 - 30 Apr 2004)	
<b>4. TITLE AND SUBTITLE</b> The Role of MEKK3 Signaling of Pathway in the Resistance of Breast Cancer Cells to TNF-(alpha)-Mediated Apoptosis			<b>5. FUNDING NUMBERS</b> DAMD17-02-1-0449	
<b>6. AUTHOR(S)</b>  Ling Yu, M.D., Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> The University of Texas M.D. Anderson Cancer Center Houston TX 77030  E-Mail: wilkie.wilson@duke.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b>  In the past year, we continued to test the hypothesis that NF- $\kappa$ B activation in breast cancer cells plays a critical role in cancer cells' resistance to anti-cancer drugs and to TNF $\alpha$ treatment, and MEKK3 is an essential component of this process. We finished construction of dominant negative forms of MEKK3 expression vectors and tested the vectors in 293T cells and in breast cancer cell line MCF7. We also prepared two lentiviral vectors for MEKK3 siRNA. The second line of this study is to further determine the regulation of NF- $\kappa$ B and AP-1 activation through MEKK3. Because both the NF- $\kappa$ B and AP-1 pathways are crucial in regulating cell apoptosis, the balance of their activation will determine the outcome of cell survival or apoptosis. We found that MEKK3 is crucial for NF- $\kappa$ B, JNK, and p38 but not for ERK MAPK activation. Furthermore we generated and characterized a unique antibody that recognize active MEKK3. This antibody will allow us to measure the effect of inhibitors and/or modulators of MEKK3 activity in breast cancer cells for investigation of the cancer cell sensitive to TNF $\alpha$ .				
<b>14. SUBJECT TERMS</b>  Breast Cancer				<b>15. NUMBER OF PAGES</b>  6
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b>  Unlimited	

## Table of Contents

Cover.....	
SF 298.....	1
Table of Contents.....	2
Introduction.....	3
Body.....	3
Key Research Accomplishments.....	3
Reportable Outcomes.....	3
Conclusions.....	4
References.....	4
Appendices.....	4

## Role of MEKK3 signaling pathway in the resistance of breast cancer cells to TNF $\alpha$ -mediated apoptosis

### Introduction

Breast cancer is the most commonly diagnosed malignancy and one of leading causes of death in American women. So far the chemotherapy and radiotherapy are still common treatments for breast cancer. However, the efficiency of the treatment usually has been limited because breast cancer develops resistance to chemotherapeutic drugs and radiation treatment. TNF $\alpha$  is one of the most pleiotropic cytokine acting as a cytotoxic agent against a variety of tumor cell lines and also play a role in tumor regression mediated by cytotoxic T cells. TNF $\alpha$  is released by cytotoxic T cells and significantly contributes to the local immune response to the tumor. Tumor cells including breast cancer cells were naturally or acquire resistance to TNF $\alpha$ -mediated apoptosis yet the mechanism is still not fully understood. We recently created MEKK3 knockout mice to investigate its in vivo function and demonstrated that MEKK3 plays a crucial role in TNF $\alpha$  induced NF- $\kappa$ B activation and apoptosis (Yang et al. 2001). Our study suggests that MEKK3 may be involved in breast cancer cells' resistance to TNF $\alpha$ -mediated apoptosis.

### Body

To test our hypothesis, we have been working on the conditions to alter the MEKK3 activities in normal and in breast cancer cells and then determine how TNF-responses will be affected. The strategy being used is to block the MEKK3 activity using dominant interfering mutants, inhibitors and siRNA technique in breast cancer cells. During this funding period, we constructed the dominant MEKK3 mutants and tested their effects on NF- $\kappa$ B reporter gene expression (figure 1). We also determined that MEKK3 is a specific activator of the NF- $\kappa$ B, JNK and p38 MAPKs, but not the ERK1/2 MAPK (figure 2). In addition, we tested MEKK3 specific siRNA lentiviral vectors to infect cell lines (figure 3) (Qin et al. 2003). We are still in the process of defining optimal conditions for infection. Finally, we prepared a unique antibody that recognize the activated MEKK3 but not inactive MEKK3 mutants (figure 4). This reagent will be a great asset for our investigation of MEKK3 activity.

### Key research accomplishment

The key accomplishments are listed above. In brief, we have generated most of the reagents that are crucial to test our working hypothesis and in the process of optimizing the experimental conditions for achieving our goals of our proposal work.

### Reportable outcomes

The major progress in the last funding period is that we generated a number of new reagents that will facilitate our investigation for the activity of MEKK3. We prepared an antibody that specifically detects active MEKK3 but not inactive mutant MEKK3 (fig 4). These studies will allow us to assess the endogenous MEKK3 activity in breast cancer cells and its modulation by inhibitors and dominant negative MEKK3 mutants. We are still working on the condition for gene delivery efficiency to tumor cells. We have successfully generated MEKK3 siRNA lentiviral vectors and obtained promising data showing that MEKK3 siRNA worked on endogenous MEKK3 protein albeit less effective than the co-transfected MEKK3.

## Conclusions

We have made good progress toward our planned research goal in understanding the molecular mechanism of NF- $\kappa$ B and MAPK activation through the MEKK3 pathway. In this regard, Dr. Huang published one manuscript that is related to this research (Huang et al, NI 2004). During the course of this work, we have a unexpected interruption because Dr. Huang was unable to obtain her visa extension to stay in US to finishing her work. Dr. Huang thus left the country in Dec. 2003 and her work is continued now by another postdoctoral fellow, Dr. Ling Yu, who joined our Institution from Japan on April 1st, 2004. Dr. Yu have started testing the conditions set-up by Dr. Huang and she quickly measured the expression and activities MEKK3 mutants in various cells and in the process of making retrovirus. Dr. Yu is also working on MEKK3 siRNA to inhibit the endogenous MEKK3 expression in breast cancer cells for further testing their effects on cancer cell sensitivity to TNF $\alpha$ .

## References

- Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z. G., and Su, B. (2004). Differential regulation of interleukin 1 receptor and Toll-like receptor signaling by MEKK3. *Nat Immunol* 5, 98-103.
- Qin, X.F., D.S. An, I.S. Chen, and D. Baltimore. 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci U S A* 100: 183-8.
- Yang, J., Y. Lin, Z. Guo, J. Cheng, J. Huang, L. Deng, W. Liao, Z. Chen, Z. Liu, and B. Su. 2001. The essential role of MEKK3 in TNF-induced NF-kappaB activation. *Nat Immunol* 2: 620-4.

## Appendices fig 1-fig 4

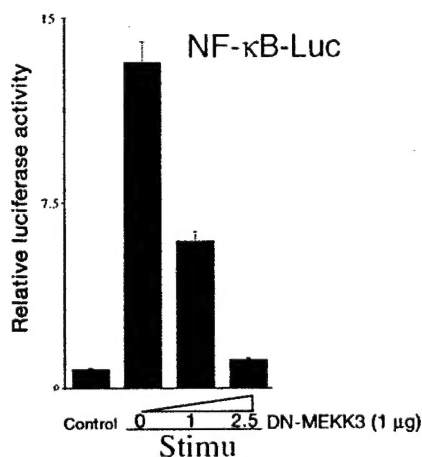


Figure 1 Dominant negative MEKK3 (DN-MEKK3) inhibits NF- $\kappa$ B reporter gene expression. One microgram of NF- $\kappa$ B-Luc reporter plasmid was transfected with either empty vector or with increasing amounts of DN-MEKK3. Transfected cells were either unstimulated (control) or stimulated (stimu) with IL-1 for 24 h before being assayed for the luciferase activity. DN-MEKK3 inhibited the reporter gene expression significantly.

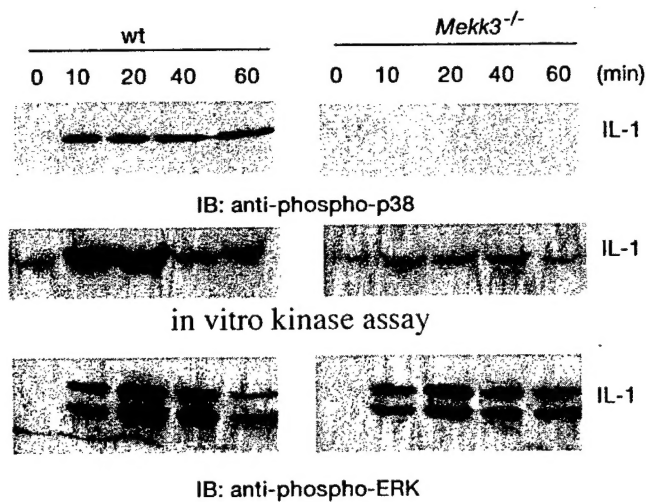


Figure 2. Activation of JNK, ERK, and p38 MAPK in wild-type and *Mekk3*<sup>-/-</sup> MEFs. Wild-type and *Mekk3*<sup>-/-</sup> MEFs either untreated or stimulated with IL-1 were harvested at the indicated time points. JNK activation was determined by an *in vitro* kinase assay, ERK and p38 MAPK activation was measured by immunoblotting with anti-phospho-p38 and anti-phospho-ERK1/2 antibodies.

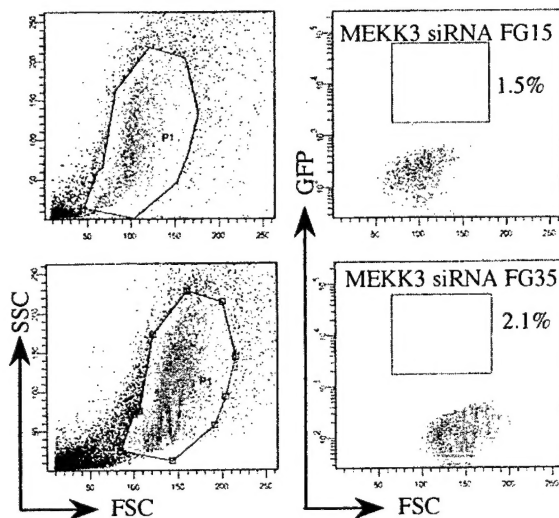


Figure 3. Infection of tumor cells with MEKK3 siRNA lentiviral expression vector. The MEKK3 siRNA cassette will be subcloned into the lentiviral vector the virus were prepared as described (Qin et al. 2003). Tumor cell line was infected with MEKK3 siRNA lentivirus FG15 and FG35, and analyzed 48 h later by FACS flow cytometry. Infected cells are shown as GFP positive. Both infections yield lesser than 3% positive cells (about 1.5 and 2.1% each).

Figure 4. Characterization of anti-phosphor-MEKK3 antibody P1. COS-1 cells were transfected with HA-tagged MEKK2, MEKK3, MEKK3 phosphorylation site mutant MEKK3A1, and DN-MEKK3 as indicated. Cells lysates were prepared 36 hr later and analyzed by immunoblotting with anti-HA antibody (top panel) and P1 antibody (bottom panel).

